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International application number: PCT/GB05/001451

International filing date: 15 April 2005 (15.04.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/521,385
Filing date: 15 April 2004 (15.04.2004)

Date of receipt at the International Bureau: 18 May 2005 (18.05.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

PA 1224229

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APPLICATION NUMBER: ***60/521,385***
FILING DATE: ***April 15, 2004***

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Electronic Version v1.1

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Title of Invention	[NEW USE AND NEW METHOD]

Application Number :

Date :

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Confirmation Number:

Attorney Docket Number: Athera2

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Description

[NEW USE AND NEW METHOD]

BACKGROUND OF INVENTION

[0001] FIELD OF THE INVENTION

[0002] This invention relates to the fields of atherosclerosis and atherothrombosis. The invention relates specifically to novel mechanisms for prevention or inhibition of atherothrombosis and plaque rupture.

[0003] DESCRIPTION OF RELATED ART

[0004] There is an interrelationship between atherosclerosis and atherothrombosis.

[0005] Atherosclerosis has many characteristics of an inflammatory disease, including abundance of inflammatory cells and production of pro-inflammatory cytokines in lesions [1,2]. The increased risk of mortality due to cardiovascular diseases, specifically in systemic lupus erythematosus (SLE) patients, is a major clinical problem.

[0006] Cardiovascular diseases in SLE patients is associated with both traditional risk factors like dyslipidemia, and non-

traditional risk factors including increased oxidation of low density lipoprotein (oxLDL), raised activity in the tumour necrosis factor (TNF)-system (closely associated with dyslipidemia), systemic inflammation as determined by CRP, homocystein and anti-phospholipid antibodies (aPL)[3-7]. Anti-phospholipid may cause the anti-phospholipid antibody syndrome (APS), common in SLE patients and characterized by recurrent pregnancy loss and recurrent thrombosis[8,9]. Different forms of anti-phospholipids have also been implicated in cardiovascular diseasesin the general population[10,11].

[0007] Annexins share the property of binding calcium and negatively charged phospholipids, both of which are required for blood coagulation.Recently, Annexin V has been implicated in anti-phospholipid antibody syndrome since some anti-phospholipid antibodies disrupt the Annexin-V antithrombotic shield in the placenta, predisposing to placental micro thrombosis and recurrent miscarriage[12-14].

[0008] The binding of Annexin V to activated platelets and to damaged cells probably explains the selective retention of the protein in thrombi. This has been shown in experimental animal models of venous and arterial thrombosis[15] and labelled Annexin has been proposed for medical

imaging of vascular thrombi in humans, with reduced noise and increased safety [16] A major problem associated with the therapeutic use of Annexin V in coagulation disorders is its short half-life in the circulation, estimated in experimental animals to be 5 to 15 minutes[15],[17] ; Annexin V has a short half-life in the circulation of humans.

[0009] In EP1379266 "Modified Annexin proteins and methods for preventing thrombosis" is claimed a polyethylene glycol-modified Annexin protein to be used to prevent thrombosis without increasing haemorrhage. The half-life of Annexin V is improved by using a PEG conjugate in a method to prevent binding of the prothrombinase complex necessary for thrombus formation.

[0010] In the present invention we have shown that Annexin V may stabilize atherosclerotic plaque. The invention is not related to blood coagulation. When Annexin V or an N-terminal fragment of Annexin V is administered according to the invention, preferably by injection, it will bind to the endothelial plaque on a first passage. The short half-life of Annexin V in the circulation is thus not a problem as is the case in EP1379266. A composition for injection comprising Annexin V or an N-terminal fragment of Annexin V with or without additives will thus prevent atherothrom-

bosis by stabilizing the carotid plaque through an instant binding.

[0011] Immunoglobulin G or IgG is a protein found in adults in normal concentrations ranging from 900 mg/dl to 2400 mg/dl. This is approximately 20% of the total protein found in serum or plasma. IgG has a half life of 23 days. It contributes immunity to bacteria, viruses, parasites, some fungi and provides antibody activity in tissue. IgG is able to activate complement which triggers the inflammatory response and is a subject unto itself. IgG provides protection to various viruses and such for the animal through inoculations or natural barnyard exposure. Once exposure occurs a memory is developed which allows they body to rapidly manufacture needed antibodies to fight specific infections. In man IgG is transferred to the baby through the placenta. If IgG is not present or in low levels, mammals have poor defences against any infectious agent to which it might be exposed.

[0012] Intravenous immunoglobulin preparations (e.g. IGIV; Baxter) is a highly purified preparation of IgG commercially available and is used in the treatment of patients who have no, or very low levels of antibody production.

[0013] Ivlg preparations from pooled plasma derived from many

donors is often used also in autoimmune conditions, where one recognized mode of action is the presence of anti-idiotypic antibodies, e g antibodies reacting with and neutralizing other antibodies that are pathogenic.

[0014] In US6613328 is described a method for treating thrombosis diseases with von Willebrand factor specific antibodies. Humanized antibodies are specifically produced. There is however no information that readily available immunoglobulins such as IGIV or subfractions of these immunoglobulins can be used to prevent atherothrombosis or plaque rupture. Furthermore, there is no information in the literature that IgG can inhibit the binding of antibodies to native Annexin V, which is a possible reason for the decreased Annexin V-binding to endothelium, presented in this invention.

SUMMARY OF INVENTION

[0015] The invention provides new methods of preventing atherothrombosis and plaque rupture and treatment of atheroscerosis complications by administrating of a compound that restores the binding of Annexin V to plaque by inhibiting Ig responsible for inhibiting the binding. This treatment is accomplished either by giving a dose of Annexin V or an N-terminal fragment, preferably by IV injec-

tion, or by IV administration of immunoglobulins or a subfraction of immunoglobulins that act to inhibit the Annexin V binding to antibodies. Immunoglobulin (IGIV; Baxter) or other commercially available preparations as well as affinity purified subfractions, which are known by the art for treatment or prevention of infections and severe autoimmune conditions, can be used. An affinity purified subfraction of immunoglobulins is preferable.

[0016] In the methods of the invention, the active component (Annexin V, N-terminal fragments of Annexin V or immunoglobulin subfraction) is administered to a subject at risk of artherothrombosis using a pharmaceutical composition having an effective amount of the active component.

[0017] The pharmaceutical composition can be administered intravenously or by other routes as a treatment of patients belonging to a risk group. A preferable risk group is systemic lupus erythematosus (SLE) patients. The treatment can be repeated at optimal time intervals.

DETAILED DESCRIPTION

[0018] The risk of atherothrombosis and plaque rupture is strongly raised when Annexin V binding to endothelium is decreased as a consequence of antibodies inhibiting the

Annexin V-plaque binding. A restoring of the Annexin V binding by administration of Annexin V (or fragments) or by administering immunoglobulins, preferably a subfraction of immunoglobulins, that inhibit other Annexin-V-binding antibodies that decrease the plaque-Annexin binding is therefore novel proposed therapies for atherothrombosis and especially plaque rupture, the main cause of cardiovascular disease.

[0019] EXPERIMENTAL

[0020] Study group

[0021] The study group consisted of 26 women with SLE who had survived one or more manifestations of cardiovascular disease, defined as thromboembolic, not hemorrhagic or vasculitic stroke (n=15), (confirmed by computed tomography or magnetic resonance imaging); myocardial infarction (n=7), (confirmed by electrocardiography and a rise in creatine kinase); angina pectoris (n=9) (confirmed by exercise stress test) or intermittent claudication (n=4) (peripheral atherosclerosis confirmed by angiogram), 26 age-matched women with SLE and no clinical manifestations of cardiovascular disease and 26 age-matched healthy population-based women.

[0022] All patients fulfilled the 1982 revised criteria of the American Rheumatism Association for SLE16. The study was approved by the Ethics Committee of the Karolinska Hospital. All participants gave informed consent before entering the study.

[0023] Carotid Ultrasound

[0024] The right and left carotid arteries were examined with a duplex scanner (Acuson Sequoia, Mountain View, California, USA) and the degree of atherosclerosis was determined by intima-media thickness (IMT) was determined.

[0025] Culture of endothelial cells binding of Annexin V

[0026] Cryopreserved pooled human umbilical venous endothelial cells (HUVECs) at passage 2 were purchased from Cascade Biologics, Inc. (Portland, OR, USA) the cultures were maintained in EGM phenol red-free medium (Clonetics, San Diego, CA, USA), containing 2% of fetal bovine serum and supplements. The cells were incubated in 75 cm2 flasks (TPP, AG, Trasadingen, Switzerland) under humidified 5% CO2 in 37°C conditions. All experiments were performed at passage 3 to 4. HUVECs were seeded at 2x104 cells / ml density in 12-well plates (NUNC, Inc, Naperville, IL, USA) for flow cytometry analysis; at density of 1x104 cells

/well/100 µl in 96-well plate (TPP) for MTT assay; at 8x103 cells/ml density in 24-well plates (NUNC) for DNA fragmentation ELISA. After allowing 12-24 hours for attachment and careful washing with serum-free medium (SFM), the cells were made quiescent in SFM for at least 12 hrs prior to treatment. Heparin-preserved plasma from the study groups was added to the monolayer at concentration of 10% in SFM.

[0027] The cells were harvested non-enzymatically with Cell Dissociation Solution (CDS; Sigma-Aldrich, St. Louis, MO, USA). HUVECs were carefully pooled with supernatants, to exclude selective loss of detached floating EC, and centrifuged at 1200 rpm for 7 min. After resuspension in 100µl of Annexin V-binding buffer (Molecular Probes Inc, Eugene, OR, USA) samples were stained with 5mg/ml of Annexin V-FITC (Mol. Probes) and incubated for 15 min on ice. Shortly before acquisition 1mg/ml of propidium iodide (PI; R&DSystems Europe Ltd, Abingdon, UK) was added. Analysis was performed on a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with CELLQUEST software. During acquisition a gate was set to exclude events smaller than 230 on linear FCS and SSC scale. For each sample 10000 events were collected.

[0028] Immunohistochemical staining of human atherosclerotic plaque

[0029] Immunostaining was performed on human plaques, characterized previously. Plaques were collected from 12 patients undergoing carotid endarterectomy after transient ischemic attacks. All specimens contained advanced atherosclerotic lesions. As a control macroscopically healthy mesenteric artery was obtained after unrelated bowel resection. The cryostat sections were fixed for 20 minutes in 2% paraformaldehyde in PBS (Sigma Chemicals) at 4°C and stored at -70°C. After blocking endogenous peroxidase, the sections were incubated overnight with monoclonal anti-Annexin V antibody (Alexis Biochemicals, Corp., Lausen, Switzerland) of mouse type IgG2a, anti-CD68 (DakoCytomation, Glostrup, Denmark) or anti-CD31 (Monosan, Uden, TheNetherlands). Irrelevant mouse IgG2a (Serotec Ltd, Oxford, UK) served as negative control. All antibodies were diluted in 1%BSA-0.02%NaN3in PBS. After washing, 1% normal horse serum in PBS was used. Secondary antibody- biotinylated horse anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA, USA) was added. The ABC peroxidase ELITE kit was used (Vector Laboratories). The staining was revealed with di-

aminobenzidine (Vector Laboratories) and counterstaining was done with haematoxyline. All sections were analyzed on a Leica DMRXA microscope (Leica, Wetzlar, Germany).

[0030] RESULTS

[0031] Effect of immunoglobulin IgG depletion on Annexin V-binding Depletion of IgG subclass of immunoglobulins resulted in up to 2.7-2.6 fold increase in fluorescence intensity of Annexin V-binding (complete serum mean FI :267.95± vs. 709.91±; median FI: 222.67± vs 567.42±). Reconstitution of IgG fraction to the depleted sera decreased the fluorescence intensity while the culture with IgG eluate resulted in fluorescence intensity of Annexin V binding comparable with that of complete sera.

[0032] Binding of Annexin V was significantly lower after 24 hrs when plasma from SLE cases was used as compared to controls (SLE cases vs population controls: $p=0.002$, SLE cases vs SLE controls $p=0.02$). Depletion of total IgG from sera with a high capacity to inhibit binding of Annexin V restored this binding completely. There was a striking positive association between Annexin V-binding and degree of atherosclerosis ($R=0.73$, $p<0.001$) among SLE cases. Immunostaining revealed presence of Annexin V in 11/12 plaques tested.

[0033] Protein G affinity column chromatography

[0034] Pooled sera with a high ability to inhibit Annexin V binding to EC were 0.45µm filtered and diluted with equal volumes of endothelial basal medium. The HiTrap Protein G HP, 1ml column with binding capacity 25 mg human IgG / ml gel from Amersham Biosciences (Uppsala, Sweden) was used according to manufacturer"s instructions. The IgG fraction was obtained by eluting the column with 0.1M glycine-HCl, pH 2.7. For neutralization 1 M Tris-HCl, pH 9.0 was used. Complete serum, effluate and eluate were used for incubation with HUVEC on the day of separation at 1:10 dilution in SFM.

[0035] In conclusion, Annexin V is present in atherosclerotic lesions at many sites, especially those that are prone to plaque rupture. When Annexin V binding is not optimal but instead decreased as a consequence of antibodies interfering with Annexin-plaque binding, the risk of atherothrombosis and plaque rupture is strongly raised. The restoring of the Annexin V- binding is therefore a possible novel therapy for atherothrombosis and especially plaque rupture, the main cause of cardiovascular disease. Two methods are provided by this invention. One is based on the use of an optimal dose of Annexin V or a

salt of the protein, which preferably should be administered by intravenous injections, the other is based on the use of ready available immunoglobulins, or an affinity purified subfraction of the immunoglobulins, which can also be administered by injections.

[0036] The effective amount of Annexin V in the dose is determined from a diagnostic status analysis on the current Annexin V- plaque binding. The binding was determined using the analysis method given above.

[0037] The treatment using a pharmaceutical composition comprising the active component is preferably administered to subjects at risk. A subject at risk is SLE patients with frequent repeating atherothrombosis.

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Claims

[c1] 1.Use of the Annexin V protein or an N-terminal fragment of Annexin V, optionally in the form of a salt, for manufacturing of a pharmaceutical composition to prevent atherothrombosis and/or plaque rupture.

[c2] 2.The use according to claim 1 wherein the pharmaceutical composition comprises an effective amount of the Annexin V protein or N-terminal fragment of Annexin V, optionally in combination with a carrier and additives.

[c3] 3.The use according to claim 1 and 2 wherein the effective amount of the Annexin V in the pharmaceutical composition is determined from a diagnostic status analysis of the Annexin V-endothelium binding.

[c4] 4.A method of treating a subject at risk of atherothrombosis and/or plaque rupture, comprising administering to said subject a pharmaceutical composition comprising an effective amount of Annexin V or its corresponding salt.

[c5] 5.A method according to claim 4 where the subject at risk is a systemic lupus erythematosus (SLE) patient.

[c6] 6.A purified subfraction of immunoglobulins with the capacity to inhibit antibodies binding to Annexin V.

[c7] 7.Use of the purified subfraction according to claim 6 or use of a commercially available immunoglobulin preparation for the manufacturing of a medicament to prevent atherothrombosis and/or plaque rupture.

[c8] 8.A pharmaceutical composition comprising an effective amount of immunoglobulins or a purified subfraction of immunoglobulins for preventing atherothrombosis and/or plaque rupture.

[c9] 9.A method of treating a subject at risk of arthothrombosis and/or plaque rupture, comprising administering to said subject a pharmaceutical composition comprising an effective amount of immunoglobulins or a purified subfraction of immunoglogulins.

[c10] 10.A method according to claim 9 where the subject at risk is a systemic lupus erythematosus (SLE) patient.

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[NEW USE AND NEW METHOD]

Abstract

Atherosclerosis can be viewed as a response to injury and it is not atherosclerosis per se that is serious but instead factors leading to rupture of atherosclerotic plaques. In the present invention we have identified such factors. A decreased binding of Annexin V to the endothelium was seen in patientswith a history of atherothrombosis. The use of native Annexin V or an N-terminal fragment as an active component or a subfraction of immunoglobulins to manufacture a pharmaceutical composition is proposed to improve said binding. The use of Annexin V, N-terminal fragments or immunoglobulins to increase the Annexin V-binding to carotid plaque represents novel mechanisms for preventing atherothrombosis.